Antigen-Specific Lymphocyte Proliferation and Interleukin Production in Chickens Immunized with Killed Salmonella enteritidis Vaccine or Experimental Subunit Vaccines

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Antigen-Specific Lymphocyte Proliferation and Interleukin Production in Chickens Immunized with Killed Salmonella enteritidis Vaccine or Experimental Subunit Vaccines

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SUMMARY. Lymphocyte proliferation and interleukin (IL)-2 and IL-6 levels in serum were measured as indicators of cell-mediated immunity after immunization of chickens with a commercial killed *Salmonella enteritidis* (SE) vaccine or experimental subunit vaccines of crude protein (CP) extract or the outer membrane protein (OMP). Significantly increased proliferative responses to SE flagella, but not lipopolysaccharide, porin, CP, or OMP, were observed at 1 wk postimmunization in the three vaccination groups. The responses to flagella were specific because flagella-induced proliferation was not seen in chickens immunized with adjuvant alone. Of the three immunization protocols, use of the killed SE vaccine appeared most effective because it induced higher flagella-stimulated lymphocyte proliferation at 1 and 2 wk postvaccination compared with the CP- and OMP-vaccinated groups. Significantly increased IL-2 and IL-6 levels in serum were seen at 1 wk postimmunization in the three vaccination groups compared with adjuvant alone, but there were no differences between the killed vaccine and the subunit vaccines at this time, and the levels of both lymphokines returned to baseline at 2 wk postimmunization. We conclude that cell-mediated immunity to SE after vaccination with the killed bacterial vaccine or subunit vaccines is transient and mainly limited to flagella.

RESUMEN. Proliferación linfocítica específica del antígeno y producción de interleucina en aves inmunizadas con una vacuna inactivada de Salmonella enteritidis o con vacunas experimentales de subunidades de Salmonella enteritidis.

La proliferación linfocítica y los niveles de interleucina 2 e interleucina 6 en el suero fueron determinados y empleados como indicadores de la inmunidad celular después de la inmunización de aves con una vacuna comercial inactivada contra *Salmonella enteritidis* o con vacunas experimentales de subunidades de extractos de proteína cruda o de la proteína de la membrana externa. Se observó un incremento significativo en la respuesta proliferativa a la proteína flagelar de la *S. enteritidis*, mas no al lipopolisacárido, porina, proteína cruda o a la proteína de la membrana externa en los tres grupos vacunados una semana después de la inmunización. La respuesta observada fue específica a la proteína flagelar ya que no se observó proliferación linfocítica en aves inmunizadas únicamente con el adyuvante. De los tres protocolos de inmunización, el uso de la vacuna inactivada contra la *S. enteritidis* pareció ser el más efectivo ya que indujo una mayor proliferación linfocítica estimulada por la proteína flagelar una y dos semanas después de la inmunización al ser comparada con los grupos vacunados con el extracto de proteína cruda o con la proteína de la membrana externa. Se observó un incremento significativo en los niveles de interleucina 2 e interleucina 6 en el suero en los tres grupos vacunados una semana después de la inmunización al ser comparado con el grupo en el cual se empleó únicamente el adyuvante. Sin embargo, no se observaron diferencias entre la vacuna

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inactivada y las vacunas de subunidades de *S. enteritidis* durante este tiempo mientras que los niveles de ambas linfocinas retornaron a sus niveles normales dos semanas después de la inmunización. Se concluye que la inmunidad celular contra *S. enteritidis* después de la vacunación con la vacuna inactivada o con vacunas de subunidades es pasajera y se encuentra limitada principalmente a la proteína flagelar.

Key words: chicken, *Salmonella*, cell-mediated immunity, interleukin, flagella

Abbreviations: Con A = concanavalin A; CP = crude protein; ELISA = enzyme-linked immunosorbent assay; HBSS = Hanks balanced salt solution; IFA = incomplete Freund adjuvant; IL = interleukin; LPS = lipopolysaccharide; OMP = outer membrane protein; PBS = phosphate-buffered saline; RPMI-10 = RPMI 1640 containing 10% fetal calf serum and antibiotics; SE = *Salmonella enteritidis*

Salmonellosis is a major cause of the human food poisoning worldwide (3,22,46). Some of the main sources of human infection are poultry products such as eggs contaminated with *Salmonella enteritidis* (SE), and recent outbreaks of salmonellosis have been epidemiologically linked with egg contamination (21,50). However, detection and elimination of SE from commercial poultry flocks have been difficult because infected chickens lay contaminated eggs at low frequency and without apparent clinical symptoms (27).

Whereas much effort has been made to develop effective control strategies against SE infection in chickens, including immunization with SE vaccines, all current methods have limitations. Live attenuated vaccines have been used with satisfactory results, but the potential for reversal to virulence through horizontal gene transfer remains a concern (5). On the other hand, killed SE vaccines may retain potentially adverse components such as bacterial lipopolysaccharide (LPS) (2), and, in general, their use has not elicited convincing levels of protection against field strains of SE (4,17,18,42,52,54). These issues have sparked interest in the development of subunit vaccines through identification of immunogenic bacterial components (37). Some researchers have suggested a subunit vaccine based on the outer membrane protein (OMP) of SE might be effective because it elicited a high level of bacteria-reactive antibodies (8,9,10). However, limited information is available on the ability of subunit vaccines to stimulate cell-mediated immunity against SE in chickens primarily because of the lack of immunologic reagents necessary for assessing avian T-cell and cytokine responses (29). Therefore, we undertook the current study to evaluate a commercial killed SE vaccine and two experimental subunit vaccines incorporating a crude protein (CP) extract or OMP with the use of reagents and methodologies developed in our laboratory to measure avian cell-mediated immunity (34,39, 49,59).

MATERIALS AND METHODS

Chickens. Seventy-five specific-pathogen-free inbred white leghorn SC chickens from Hy-Vac (Adel, IA) were obtained as fertilized eggs, hatched, and maintained in floor pens at the Animals and Natural Resources Institute (Beltsville, MD) according to guidelines of the Beltsville Agriculture Research Center Small Animal Care Committee.

Preparation of SE components. *Salmonella enteritidis* strain FDA 338 was grown in trypticase-soy broth supplemented with yeast extract at 37 C overnight. To prepare the CP extract, bacteria in log-phase growth were washed two times with phosphate-buffered saline (PBS) by centrifugation at 4500 × *g* for 30 min at 4 C, adjusted to 10^9–10^{10} colony-forming units/ml, heated for 10 min at 100 C, and sonicated on ice for 6 min. To prepare OMP, 100 ml of log-phase SE was resuspended in PBS, sonicated on ice for 3 min, and centrifuged for 30 min at 1500 × *g* at 4 C. The resulting supernatant was centrifuged for 30 min at 20,000 × *g*, and the pellet was used as OMP. To prepare flagella, bacteria were homogenized in PBS for 30 sec at 30,000 rpm with a tissue demembranator (OMNI International, Warrenton, VA), the homogenate was centrifuged at 2000 × *g* for 30 min at 4 C, and the supernatant was used as flagella. To prepare porin, bacteria were resuspended in PBS containing 2% sodium dodecyl sulfate and centrifuged at 2000 × *g* for 30 min at 4 C, and the resulting supernatant was centrifuged at 20,000 × *g* for 30 min at 4 C to produce a clear gel-like pellet containing porin. Protein concentrations were measured by the method of Lowry *et al.* (35).

Immunizations. Chickens were separated into five groups (15 animals/group) and immunized subcutaneously in the neck at 6 and 9 wk of age as follows. Group 1 received 0.3 ml of a commercial killed SE vaccine (Poulvac® SE; Fort Dodge Animal Health, Overland Park, KS), group 2 received 1.0 ml of CP

extract containing 0.5 mg mixed 1:1 with incomplete Freund adjuvant (IFA) (Sigma, St. Louis, MO), group 3 received 1.0 ml of OMP containing 0.5 mg in IFA, group 4 received 1.0 ml of saline in IFA, and group 5 received 1.0 ml of saline alone.

Cell proliferation assay. Spleens were collected at 1, 2, and 3 wk after the first immunization and 1 and 2 wk after the second immunization. At each time point, three chickens from each group were euthanatized and their spleens were gently pressed through cell strainers (Becton Dickinson, Franklin Lakes, NJ) with glass syringe plungers into conical tubes containing Hanks balanced salt solution (HBSS) (Sigma). Cell suspensions from the three chickens of each group were pooled, washed twice, resuspended in HBSS, layered onto Histopaque®-1077 (Sigma), and centrifuged according to the manufacturer's instruction. Cells at the interface were collected, washed twice, and resuspended in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Sigma) (RPMI-10). Cell viability was determined by trypan blue exclusion and 2.5×10^6 cells were added to wells of 96-well cell culture plates containing 100 μl of RPMI-10 medium alone or medium containing 2.0 μg/ml of concanavalin A (con A), SE LPS (Sigma), CP, OMP, porin, or flagella. The cells were incubated for 48 hr at 41.5 C in 5% CO_2, and cell proliferation was determined with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) as described (40). Optical densities at 450 nm were measured by an automated microplate reader (Bio-Rad, Richmond, CA). Each assay was performed in triplicate.

Interleukin (IL)-2 and IL-6 enzyme-linked immunosorbent assay (ELISA). The direct binding IL-2 and IL-6 ELISAs were performed as described (34,49). Briefly, serum samples were adsorbed to 96-well microtiter plates, and the plates were washed with PBS containing 0.05% Tween-20 (Sigma) and blocked with PBS containing 1% bovine serum albumin. Bound IL-2 or IL-6 was detected by sequential incubations with 100 μl/well of a predetermined concentration of monoclonal antibody against chicken IL-2 (38) or IL-6 (H. Lillehoj, unpubl. data), 50 μl of peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (1:2000; Sigma), and 100 μl of 0.01% 3,3′,5,5′-tetramethylbenzidine dihydrochloride (Sigma). Optical densities at 450 nm were measured by an automated microplate reader (Bio-Rad). Each assay was performed in quadruplicate on three chickens from each of the five time points postvaccination.

Statistical analysis. Mean values for lymphocyte proliferation and IL-2 and IL-6 levels were calculated and differences were analyzed by one-way ANOVA with GraphPad InStat® Software (San Diego, CA) or the procedure of Tukey and considered significant at $P < 0.05$. For lymphocyte proliferation, we compared mean values among the medium control, con A, LPS, flagella, porin, CP extract, and OMP at each of the five time points postvaccination and within each of the five vaccination groups. In addition, we compared the proliferative responses to flagella among vaccination groups at each time point. For serum IL-2 and IL-6 levels, we compared mean values among the five vaccination groups at each of the five time points postvaccination.

RESULTS

Lymphocyte proliferation. As shown in Fig. 1, lymphocytes from chickens immunized with the commercial killed SE vaccine (group 1) demonstrated significantly increased proliferation to SE flagella at 1 wk postvaccination compared with LPS, porin, CP extract, or OMP ($P < 0.05$). Indeed, the response to flagella was equal to the con A positive control at this time. At 2 wk postvaccination, however, the response to flagella in group 1 chickens was significantly less than the con A response but still greater than that to LPS, porin, or CP extract. By 3 wk postimmunization, the proliferative response to flagella had decreased to the levels seen with the other SE antigens.

Lymphocytes from chickens vaccinated with the CP extract (group 2) exhibited significantly increased flagella-stimulated proliferation at 1 wk postimmunization compared with medium control, LPS, or CP ($P < 0.05$) but not porin or OMP. At 2 and 3 wk postimmunization, group 2 birds did not exhibit an increased response to flagella compared with the medium control. Lymphocyte proliferation to flagella in OMP-vaccinated birds (group 3) was significantly greater than the medium control only at 3 wk postimmunization although results from the 2-wk time point were clouded by the fact that proliferation to the con A positive control was not seen at this time. Also, unlike group 1 chickens, the response to flagella was significantly less than that to con A in groups 2 and 3 chickens at 1 wk postimmunization.

When lymphocyte proliferative responses to flagella were compared among the three different vaccination groups at the individual time points, chickens given the killed SE vaccine exhibited significantly greater proliferation compared with CP- and OMP-vaccinated birds ($P < 0.05$) at 1 wk, but not at 2 or 3 wk postvaccination. Lymphocytes from chickens given IFA (group 4) or saline alone (group 5) showed no proliferation to any of the SE antigens at any of the time points examined. Finally, to assess their secondary responses, the three vac-

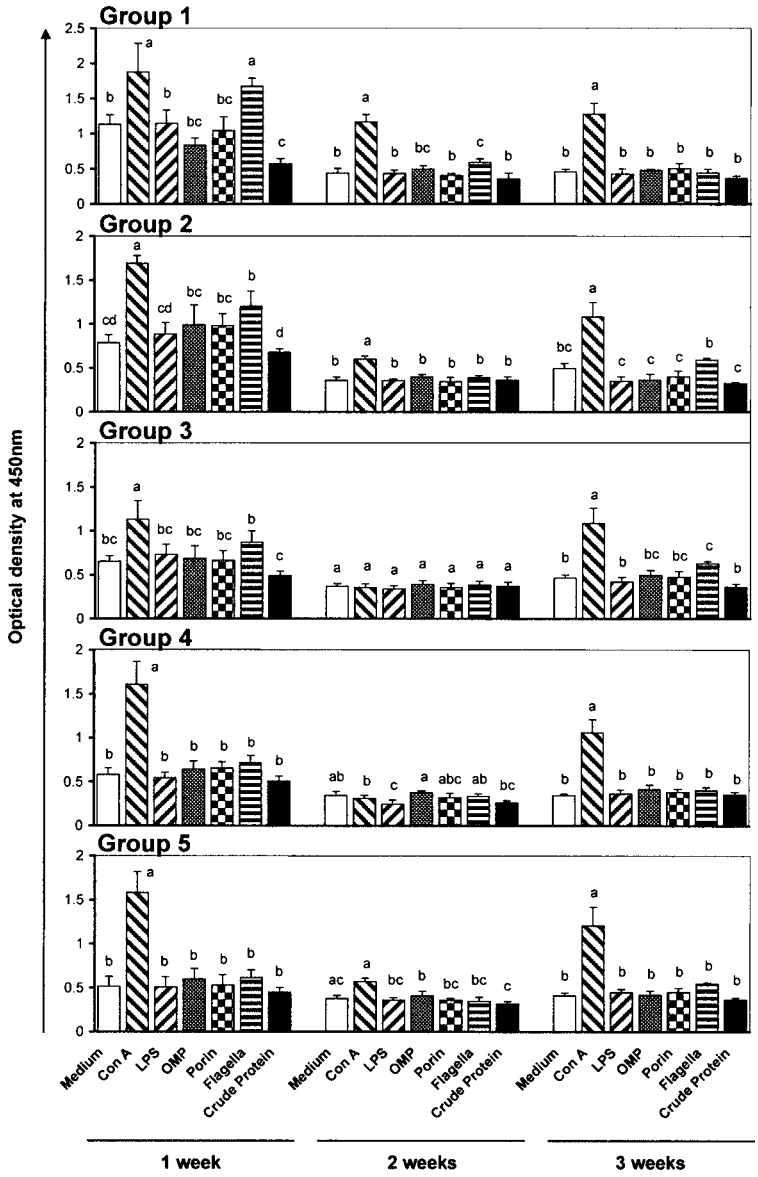

Fig. 1. Lymphocyte proliferation in chickens immunized with killed SE vaccine (group 1), CP extract (group 2), OMP (group 3), IFA (group 4), or saline (group 5). Spleen cells were isolated at 1, 2, or 3 weeks post-vaccination and stimulated with medium alone (□) or 2.0 μg/ml of con A (▨), LPS (▨), OMP (▦), porin (▦), flagella (▤), or CP (■) as described in the Materials and Methods. Values represent the mean ± SD of spleen cells pooled from 3 chickens and performed in triplicate. Columns within treatment groups with no common lowercase letters differ significantly ($P < 0.05$) when compared at each time point.

cination groups were given a booster vaccination at 3 wk after their primary immunization and lymphocyte proliferation was determined 1 and 2 wk later. However, no proliferative response to any of the SE antigens was seen in any group after the secondary immunizations (data not shown).

Serum IL-2 and IL-6 levels. As shown in Fig. 2, serum IL-2 and IL-6 levels were significantly greater at 1 wk after vaccination with the killed SE vaccine or the CP or OMP subunit vaccines compared with the IFA or saline control groups. No difference in either lymphokine was apparent

among the three vaccine groups at this time point. Similarly, no difference among any of the five groups was seen at 2 and 3 wk post primary immunization or 1 and 2 wk post secondary immunization (4 and 5 wk post primary immunization).

DISCUSSION

In this study, we measured lymphocyte proliferation and interleukin production in chickens immunized with a commercial killed SE vaccine or experimental subunit vaccines prepared from a CP extract or bacterial OMP. On the basis of results from the proliferation assay, we conclude that the majority of the cell-mediated response was directed against SE flagella. Furthermore, by both proliferation assay and interleukin production, cellular immunity to SE was relatively short lived after primary vaccination and was not detected after secondary immunization in the three vaccination groups. Finally, immunization with the killed SE vaccine was superior to the subunit vaccines because flagella-stimulated proliferation at 1 and 2 wk postimmunization in the killed vaccine group was significantly greater compared with the CP- and OMP-vaccinated animals. Indeed, chickens immunized with the killed vaccine exhibited a proliferative response to flagella equal to that of the con A T-cell mitogen within 1 wk after vaccination.

Previously, delayed type hypersensitivity was used as an indicator of T-cell-mediated immunity in avian salmonellosis (19,20). However, delayed type hypersensitivity is complicated by possible Arthus reaction and nonspecific factors related to LPS activation (30,36). Moreover, measurement of spleen cell mitogen responses in *Salmonella*-infected chickens (1) is not a reliable indicator of antigen-specific T-cell responses during salmonellosis. Because antigen-specific T-cell proliferation and lymphokine assays provide a better indication of cell-mediated immunity, we and others (28,32,55) consider these parameters better suited to assess cellular immunity in chickens immunized with different SE vaccines.

IL-2 is the prototypical Th1-type cytokine and a central mediator of cell-mediated immunity in avians and mammals (44,45). In addition to playing a role in acute phase reactions (31), mammalian IL-6 not only is involved in the proliferation and differentiation of T cells (11,23,26) and mucosal B cells (6,7) but also is an important component of the host's response to infection by different *Salmonella* spp. (13,14,32). Although the function of IL-6 in avian cellular immunity remains to be clarified,

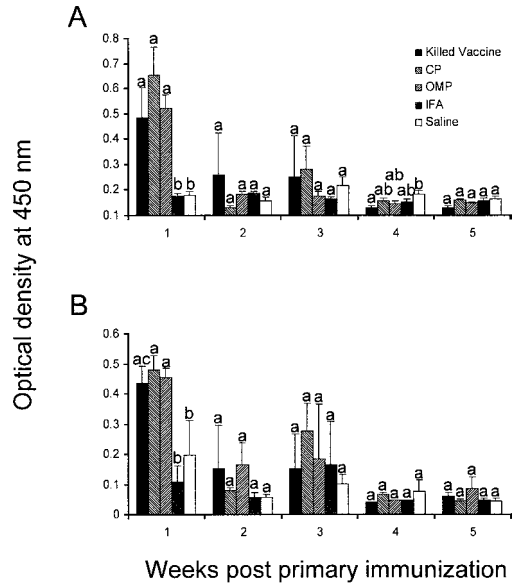

Fig. 2. Serum IL-2 (A) and IL-6 (B) levels following immunization with killed SE vaccine, CP extract, OMP, IFA, or saline. IL-2 and IL-6 were measured by ELISA as described in Materials and Methods. Values represent the mean ± SD of 3 individual chickens each performed in quadruplicate. Columns within time points with no common lowercase letters differ significantly ($P < 0.05$).

two recent studies suggested that it also plays an important role in this process. First, differential expression of avian IL-6 transcripts was proposed to be responsible for the type of clinical symptoms associated with the pathogenesis of *Salmonella* serovars (29). Second, chickens produced IL-6 in response to injection of *Salmonella typhimurium*-derived LPS (57).

A better understanding of the spectrum of immune responses induced by SE vaccines should provide useful insights into the nature of protective immunity in avian salmonellosis. A number of studies focusing on OMP proteins as subunit vaccines for SE have shown encouraging results not only in inducing high antibody titers but also in reducing the bacterial burden in internal organs and bacterial shedding in feces (8,9,10). Nevertheless, our current study demonstrates that immune effector mechanisms involving T cells and lymphokines also play a significant role in eliminating intracellular *Salmonella* from infected animals. Although we were unable to demonstrate heightened lymphocyte proliferation or IL-6 and IL-2 responses after booster vaccinations, we consider it

very possible that effector mechanisms different from those evaluated here may mediate a secondary immune response against *Salmonella*. Another possibility is the lack of an anamestic immune response may be real and responsible for chronic SE infection in chickens. Indeed, Curtiss *et al.* (12) suggested that incomplete protective immunity after *Salmonella* infection may lead to a carrier state producing systemic infection and ultimately leading to egg contamination (5,24,41,43,48,51,53).

Thus, one of the current challenges in developing an effective vaccine against avian salmonellosis is the ability to eliminate the carrier state. Although we observed that the commercial SE vaccine was better than the subunit vaccines for inducing primary cellular responses, it was no more effective in inducing a secondary cellular response compared with the CP or OMP preparations. The composition and purity of our experimental subunit vaccines remain to be determined, but it is unlikely that contaminating LPS was responsible for the observed effect on lymphocyte proliferation because purified LPS did not stimulate proliferation. Furthermore, whereas porin is a major component of OMP (25,55), porin itself was unable to induce lymphocyte proliferation in CP- or OMP-vaccinated birds. Rather, these animals, like those vaccinated with the commercial vaccine, displayed the highest proliferative response to flagella, suggesting that SE flagella might be a vaccine candidate worthy of further study. Flagella-specific ELISAs have been widely used to detect *Salmonella* antibody responses (15,16,58,60), and Wyant *et al.* (56) recently demonstrated that the flagellar antigen of *Salmonella typhi* is a powerful monocyte activator. We are currently investigating the flagella content of our OMP preparation and its potential as a subunit vaccine.

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